



# Corrective effects of hepatotoxicity by hepatic Dyrk1a gene delivery in mice with intermediate hyperhomocysteinemia



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## ABSTRACT

Hyperhomocysteinemia results from hepatic metabolism dysfunction and is characterized by a high plasma homocysteine level, which is also an independent risk factor for cardiovascular disease. Elevated levels of homocysteine in plasma lead to hepatic lesions and abnormal lipid metabolism. Therefore, lowering homocysteine levels might offer therapeutic benefits. Recently, we were able to lower plasma homocysteine levels in mice with moderate hyperhomocysteinemia using an adenoviral construct designed to restrict the expression of DYRK1A, a serine/threonine kinase involved in methionine metabolism (and therefore homocysteine production), to hepatocytes. Here, we aimed to extend our previous findings by analyzing the effect of hepatocyte-specific *Dyrk1a* gene transfer on intermediate hyperhomocysteinemia and its associated hepatic toxicity and liver dysfunction. Commensurate with decreased plasma homocysteine and alanine aminotransferase levels, targeted hepatic expression of DYRK1A in mice with intermediate hyperhomocysteinemia resulted in elevated plasma paraoxonase-1 and lecithin:cholesterol acyltransferase activities and apolipoprotein A-I levels. It also rescued hepatic apolipoprotein E, J, and D levels. Further, Akt/GSK3/cyclin D1 signaling pathways in the liver of treated mice were altered, which may help prevent homocysteine-induced cell cycle dysfunction. *DYRK1A* gene therapy could be useful in the treatment of hyperhomocysteinemia in populations, such as end-stage renal disease patients, who are unresponsive to B-complex vitamin therapy.

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## 1. Introduction

Cystathionine beta synthase (CBS) deficiency is the most common inborn error of one-carbon metabolism and is the cause of classical homocystinuria, a condition characterized by high levels of plasma homocysteine (hcy) or severe hyperhomocysteinemia (hhcy) [1]. Elevated

plasma hcy, or hhcy, is categorized by range as moderate (15 to 30  $\mu$ M), intermediate (30 to 100  $\mu$ M), and severe (above 100  $\mu$ M). Hyperhomocysteinemia is associated with increased risk for congenital disorders, including neural tube closure defects, heart defects, cleft lip/palate, Down syndrome, and multi-system abnormalities in adults [2].

hcy is a thiol-containing amino acid produced during metabolism of methionine (an essential amino acid supplied by dietary proteins) via the adenosylated compounds S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH). The metabolism of hcy occurs at the junction of two pathways: remethylation, the process leading to the reconstruction of the methionine particle, and the transsulfuration pathway. Conversion of hcy to cystathionine is catalyzed by CBS, which is vitamin B6-dependent and functions first in the transsulfuration pathway. hcy can also revert to SAH via reversal of the S-adenosylhomocysteine hydrolase (SAHH) reaction [3]. Increased hcy synthesis and its slower intracellular utilization cause increased efflux into the blood. Hence, plasma hcy level is an important reflection of hepatic methionine metabolism and

**Abbreviations:** ALT, alanine aminotransferase; APO, apolipoprotein; CBS, cystathionine beta synthase; DCP, 2,6-dichlorophenolindophenol; hcy, homocysteine; HDLs, high-density lipoproteins; hhcy, hyperhomocysteinemia; HPLC, high-performance liquid chromatography; KYNA, kynurenic acid; LCAT, lecithin:cholesterol acyltransferase; NQO1, NAD(P)H:quinone oxidoreductase; PON-1, paraoxonase-1; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; SAHH, S-adenosylhomocysteine hydrolase; VLDL, very low-density lipoprotein.

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of the rate of processes modified by B vitamins as well as activities of different enzymes [4].

Current treatment for CBS deficiency involves lowering hcy levels with a combination of vitamins, protein restriction, and cysteine supplementation [5–7]. Although treatments can be effective, the challenging dietary regimens often create problems with compliance. However, approximately 50% of patients with CBS deficiency are biochemically responsive to pyridoxine (vitamin B6) and show improvement in plasma hcy levels [8]. Therefore, drugs that could stimulate residual CBS enzyme activity could be useful in treating the disease.

One possible approach to stimulating CBS activity is by using gene therapy to deliver a gene (and, therefore, protein) that could increase the activity of existing CBS protein in the liver. We recently used an adenoviral construct designed to restrict overexpression of DYRK1A, a serine/threonine kinase involved in several steps of methionine metabolism, to hepatocytes of hhcy mouse models [9,12]. Indeed, we have demonstrated the involvement of hepatic Dyrk1a levels on hepatic SAHH and CBS activities by a mechanism dependent of NAD(P)H: quinone oxidoreductase (NQO1) activity and pyridoxal phosphate respectively [10–12]. Injection of the specific adenoviral *Dyrk1a* gene transfer construct to mice with moderate hhcy produced decreased plasma hcy levels, in agreement with restoration of key enzymes of hcy metabolism, SAHH and CBS. Pyridoxal phosphate, the metabolically active form of vitamin B6 and cofactor of CBS, was elevated, consistent with the increase in CBS activity [11].

As the central organ of metabolism, many metabolic diseases originate in the liver; however, clinical manifestations can be extrahepatic. In the case of hhcy, the liver plays not only a central role in the metabolism of methionine and contributes the high levels of plasma hcy, but also produces and degrades lipoproteins. High-density lipoproteins (HDLs) exert potent protective effects, including the prevention and correction of endothelial dysfunction by their anti-oxidative and their anti-inflammatory properties [13]. Commensurate with decreased plasma hcy levels, targeted hepatic expression of *Dyrk1a* by adenoviral gene transfer resulted in elevated activity of plasma paraoxonase-1 (PON-1), an HDL-associated protein that inactivates lipids in oxidized low-density lipoproteins, and increased plasma levels of apolipoprotein A-I (APO A-I), the major protein component of HDLs. Additionally, the Akt/GSK3 signaling pathways were rescued in the aorta of targeted mice, thus preventing hcy-induced endothelial dysfunction [11]. In the current study, we aimed to extend our previous findings by analyzing the effect of hepatocyte-specific *Dyrk1a* gene transfer on intermediate hhcy and on the associated hepatic toxicity by measuring plasma alanine aminotransferase (ALT) levels, and liver dysfunction by measuring lipid markers.

## 2. Materials and methods

### 2.1. Experimental animals

All procedures were carried out in accordance with internal guidelines of the French Agriculture Ministry for animal handling. Mice were maintained in a controlled environment with unlimited access to food and water on a 12 h light/dark cycle. Mice were fed a standard laboratory diet (CRM, Special Diets Services, Dietex, France Usine). This diet has a protein content of 19%, a methionine content of 2.700 mg kg<sup>-1</sup>, a folic acid content of 4.41 mg kg<sup>-1</sup>, and a vitamin B12 content of 0.082 mg kg<sup>-1</sup>. The number of mice and suffering were minimized as possible. Mice heterozygous for targeted disruption of the *Cbs* gene (*Cbs*<sup>+/-</sup>) were generously donated by Dr. N. Maeda (Department of Pathology, University of North Carolina, Chapel Hill, NC, USA) [12]. *Cbs*<sup>+/-</sup> mice, on a C57BL/6 background, were obtained by mating male *Cbs*<sup>+/-</sup> mice with female wild-type C57BL/6 (*Cbs*<sup>+/+</sup>) mice. DNA isolated from tail biopsies of 4-week-old mice was subjected to genotyping of the targeted *Cbs* allele using polymerase chain reaction (PCR) [14]. The E1E3E4-deleted adenoviral vector “AdDYRK1A” was constructed to

induce hepatocyte specific overexpression of DYRK1A as described previously [11]. Generation of the E1E3E4-deleted adenoviral vector “AdDYRK1A” and large-scale vector production were performed as described previously [15]. Before the experiments and to induce intermediate hhcy, female *Cbs*<sup>+/-</sup> mice, 3 at 4 months of age, were maintained for three months on the standard diet supplemented with 0.5% L-methionine (Sigma-Aldrich, France) in drinking water. Mice were divided into two groups for the last month, with one group receiving injection in the retro-orbital sinus with an adenoviral vector AdDYRK1A to have  $2 \times 10^{12}$  adenoviral particles/kg body weight and the second group receiving an equivalent dose of saline buffer [11]. Control mice, healthy control *Cbs*<sup>+/+</sup> mice also received an equivalent dose of saline buffer and were used as references to monitor hyperhomocysteinemic development. Five to twelve mice per experiment were used.

### 2.2. Preparation of serum samples, tissue collection, and plasma assays

Upon euthanization of mice by Ketamine/Xylazine intraperitoneal injection, blood samples were obtained by retro-orbital sinus sampling with heparinized capillaries, collected into tubes containing a 1/10 volume of 3.8% sodium citrate, and immediately placed on ice. Plasma was isolated by centrifugation at 2500 ×g for 15 min at 4 °C. Livers were harvested, snap-frozen, and stored at -80 °C until use. Plasma total hcy, defined as the total concentration of hcy after quantitative reductive cleavage of all disulfide bonds, was assayed using the fluorimetric high-performance liquid chromatography (HPLC) method as previously described [16]. Samples were treated with perchloric acid and kynurenic acid (KYNA) was quantified by LC-MS/MS with CTC-PAL autosampler on an Agilent 1200 series system with a quaternary pump, and MS detection was performed on an API 3200 MS/MS spectrometer (ABSciex) operated with a Turbo Ion Spray source [17]. KYNA used as standard was purchased from Sigma-Aldrich. Data were acquired and processed using Analyst software (V. 1.4.2). Plasma APO A-I protein levels were measured by ELISA (E90519M, Uscn, Life Science Inc.) according to the manufacturer's instructions. After the development of the colorimetric reaction, optical density (OD) at 450 nm was quantified by a microplate reader (Flex Station3, Molecular Device), and OD readings were converted to concentrations (µg/mL) on the basis of the standard curve obtained with APO A-I standard preparation. ALT was assayed using the Alanine Aminotransferase Activity Assay Kit (Sigma-Aldrich, France), based on the pyruvate generated.

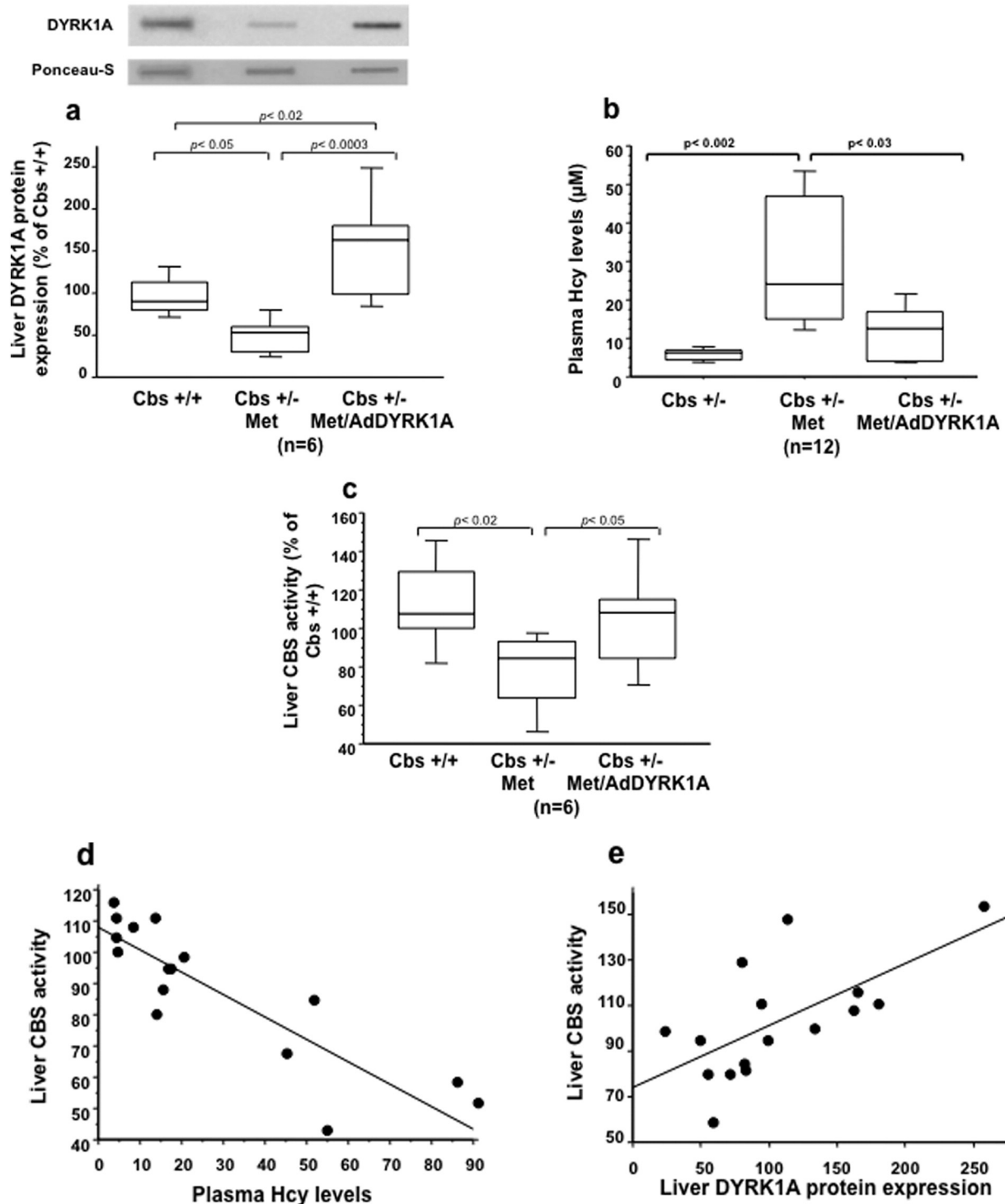
### 2.3. Protein extraction and slot blot analysis

Liver protein extracts were prepared by homogenizing tissue in phosphate-buffered saline (PBS) with a cocktail of protease inhibitors (1 mM Pefabloc SC, 5 µg/mL E64, and 2.5 µg/mL Leupeptin). Homogenates were centrifuged at 12,500 ×g for 15 min at 4 °C. Supernatants were then assayed for protein concentrations with the Coomassie (Bradford) Protein Assay reagent (Bio-Rad). Since specificity of each primary antibody used was previously validated by Western blotting, protein extracts (40 µg) under reducing conditions were subjected to slot blotting on nitrocellulose transfer membrane PROTRAN® (Whatman). The membrane was then blocked in 10% nonfat dry milk in Tris-saline buffer (1.5 mM Tris, 5 mM NaCl, 0.1% Tween-20) and probed overnight at 4 °C with one of the following antibodies: anti-APO D (1/1000; Santa Cruz Biotechnology, Tebu, France), anti-APO E (1/1000; Santa Cruz Biotechnology), anti-APO J (1/1000; Santa Cruz Biotechnology), anti-DYRK1A (1/250; Abnova Corporation, Tebu, France), anti-Akt (1/1000; Santa Cruz Biotechnology), anti-phospho-Akt1/2/3 (Ser 473; 1/1000; Santa Cruz Biotechnology), anti-GSK (1/2000, Santa Cruz Biotechnology), anti-phospho-GSK3 (ser21-9; 1/400, Cell Signaling, Ozyme, France), or anti-cyclin D1 (1/250, Cell Signaling). Horseradish peroxidase-conjugated secondary antibody and Western Blotting Luminol Reagent (Santa Cruz Biotechnology) were used to detect specific proteins. Digitized images of the immunoblots obtained using an LAS-

3000 imaging system (Fugi Photo Film Co., Ltd.) were used for densitometric measurements with an image analyzer (UnScan It software, Silk Scientific Inc.). Quantification of total proteins after Ponceau-S coloration was used as an internal control.

#### 2.4. Enzyme activity assays

CBS activity assay was performed on 300 µg of protein extracts as described previously [18]. Proteins were incubated for 1 h at 37 °C with



**Fig. 1.** Effect of hepatic overexpression of *Dyrk1a* on plasma hcy levels and hepatic CBS activity in mice with intermediate hhcy. DYRK1A protein expression in the liver (a), hcy in plasma (b), and CBS activity in the liver (c) of wild-type (Cbs<sup>+/+</sup>) mice and Cbs<sup>+/-</sup> mice supplemented with methionine and injected (Cbs<sup>+/-</sup> Met/AdDYRK1A) or uninjected (Cbs<sup>+/-</sup> Met) with AdDYRK1A. DYRK1A level was determined by slot blotting, and values were obtained by normalization of images from DYRK1A to total proteins colored with Ponceau-S. Data of DYRK1A expression and CBS activity were normalized to the mean of wild-type mice (Cbs<sup>+/+</sup>). Data correspond to the medians with interquartile ranges. n = number of mice. Levels of plasma hcy and hepatic CBS activity were negatively correlated at  $p < 0.0013$  with a  $\rho = -0.861$  for hcy levels  $> 5 \mu\text{M}$  (d). Levels of hepatic Dyrk1a expression and CBS activity were positively correlated at  $p < 0.01$  with a  $\rho = 0.655$  (e).

1 mM DL-propargylglycine, 0.2 mM pyridoxal 5'-phosphate, 10 mM L-serine, 10 mM DL-hcy, and 0.8 mM S-(5'-adenosyl)-L-methionine, using a DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] based-assay. The reaction was performed at 37 °C by measuring the absorbance at 412 nm over 10 min, using a spectrophotometer (Lambda XLS, PerkinElmer). Lecithin:cholesterol acyltransferase (LCAT) activity was assayed on 2  $\mu$ L of plasma with an LCAT kit per manufacturer's directions (Calbiochem/EMD-Millipore/Merck KGaA, Darmstadt, Germany). NAD(P)H:quinone oxidoreductase (NQO1) activity was assayed on 150  $\mu$ g of protein extracts as described [19]. Proteins were incubated for 8 min at room temperature in PBS containing 0.07% bovine serum albumin (pH 7.4) and 0.01% Tween-20. Then, a mixture containing 0.2 mM  $\beta$ -nicotinamide adenine dinucleotide, reduced (NADH), 5  $\mu$ M flavin adenine dinucleotide (FAD), and 25 mM Tris-HCl (pH 7.4) was added to the protein preparations. Two conditions were prepared, with or without 10  $\mu$ mol of dicoumarol (Calbiochem, MERCK) used to specifically block NQO1. The reaction was started by adding 40  $\mu$ M of 2,6-dichlorophenolindophenol (DCPIP). The reduction of DCPIP was assayed by measuring the absorbance at 600 nm every 30 s for 3 min using a spectrophotometer (Lambda XLS, PerkinElmer). NQO1 activity was determined by subtraction of the activity recorded in the presence of dicoumarol. PON1 activity assay was performed on 100  $\mu$ g of liver protein extracts or 5  $\mu$ L of plasma. PON1 arylesterase activity toward phenyl acetate was quantified spectrophotometrically using 20 mM Tris-HCl (pH 8.3), 1 mM CaCl<sub>2</sub>, and 10 mM phenyl acetate. The reaction was performed at room temperature by measuring the appearance of phenol at 270 nm every 10 s for 1 min using a spectrophotometer (Lambda XLS, PerkinElmer). SAHH activity was measured in the forward hydrolysis direction following the protocol described by Villanueva and Halsted [20]

with some modifications. Protein extracts (300  $\mu$ g) were incubated for 5 min at 37 °C in 1 mL of reaction mixture (452 mM NaCl, 8.9 mM KCl, 33 mM Na<sub>2</sub>HPO<sub>4</sub>, 6.6 mM KH<sub>2</sub>PO<sub>4</sub>, 0.1% gelatin, 1.2 mM MTT, 1.1 U adenosine deaminase, 0.45 U nucleoside phosphorylase, and 0.1 U xanthine oxidase). The SAHH reaction was started by adding 80  $\mu$ M S-(5'-adenosyl)-L-homocysteine to the mixture. Following 5 min of incubation at 37 °C, SAHH-specific activity was detected by the production of formazan, which is detected by measuring the absorbance at 578 nm using a spectrophotometer (Lambda XLS, PerkinElmer).

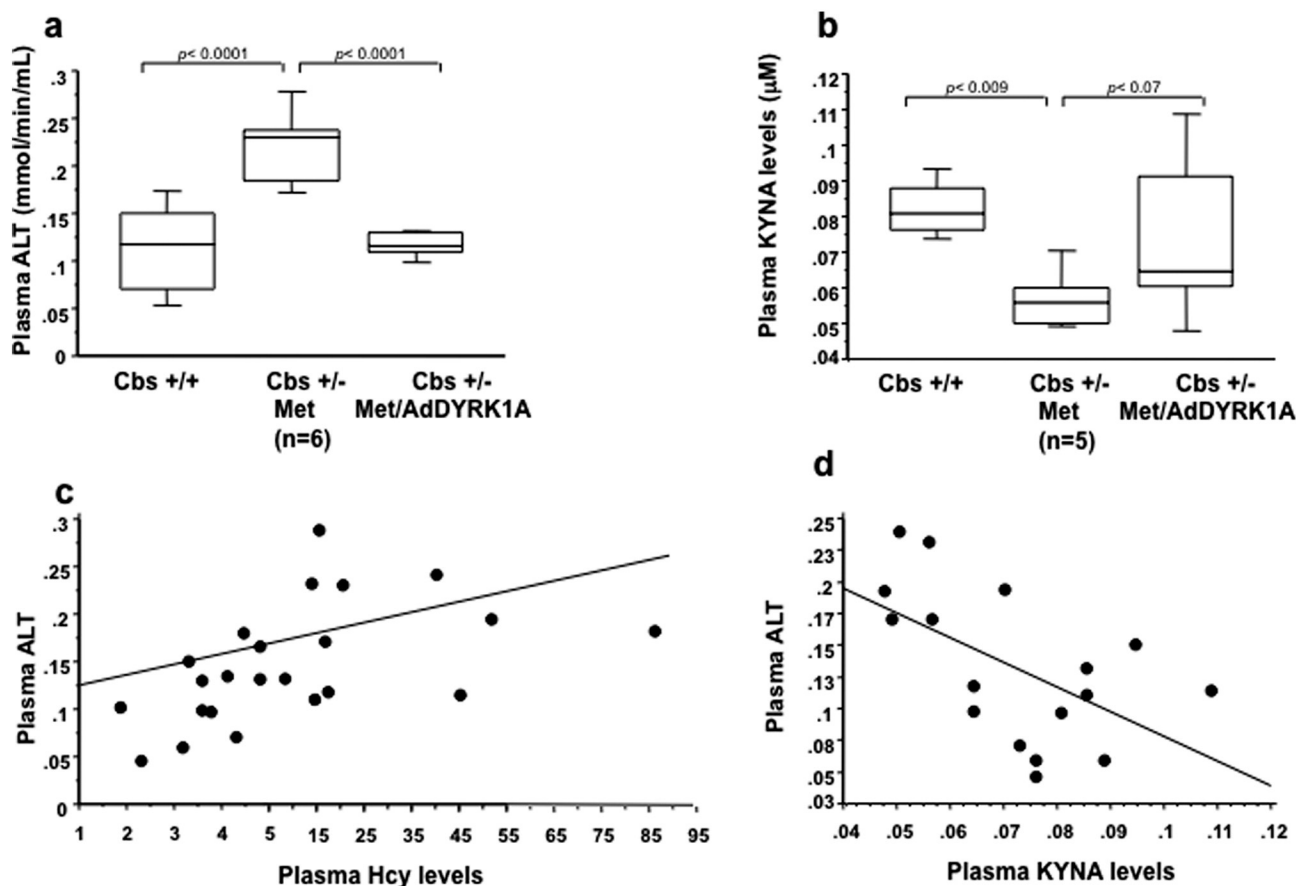
## 2.5. Data analysis

Statistical analysis was done with one-way ANOVA followed by Fisher post-hoc test using Statview software. The results are expressed as medians with interquartile ranges. Data were considered significant when  $p \leq 0.05$ . A  $p$  value of 0.06–0.10 was considered to indicate a strong statistical tendency due to the small sample size. Correlations were determined by using Spearman's rank correlation, as data were not normally distributed according to Shapiro–Wilk test.

## 3. Results

### 3.1. *Dyrk1a* overexpression reduces plasma hcy levels in mice with intermediate hhcy

To overexpress *Dyrk1a* specifically in the liver of mice, we used an E1E3E4-deleted adenoviral vector “AdDYRK1A”, which was constructed to induce hepatocyte-specific expression of *Dyrk1a* [11]. We injected “AdDYRK1A” into CBS-deficient mice ( $Cbs^{+/-}$ ) supplemented with



**Fig. 2.** Effect of hepatic overexpression of *Dyrk1a* on plasma ALT and KYNA levels in mice with intermediate hhcy. ALT (a) and KYNA (b) in plasma of wild-type (*Cbs*<sup>+/+</sup>) mice and *Cbs*<sup>+/-</sup> mice supplemented with methionine and injected (*Cbs*<sup>+/-</sup> Met/AdDYRK1A) or uninjected (*Cbs*<sup>+/-</sup> Met) with AdDYRK1A. Data correspond to the medians with interquartile ranges.  $n$  = number of mice. Levels of plasma ALT and hcy were positively correlated at  $p < 0.011$  with a  $\rho = 0.543$  (c). Levels of plasma ALT and KYNA were negatively correlated at  $p < 0.024$  with a  $\rho = 0.563$  (d).



methionine in drinking water, a murine intermediate model of hhcy. Similar to a previous report [9], mean DYRK1A protein level was decreased in the liver of  $Cbs^{+/-}$  mice supplemented with methionine when compared with wild-type ( $Cbs^{+/+}$ ) mice (Fig. 1a). However, after injection of AdDYRK1A to  $Cbs^{+/-}$  mice supplemented with methionine, we observed an increase in DYRK1A protein levels when compared with  $Cbs^{+/-}$  mice supplemented with methionine but uninjected (Fig. 1a).

To determine the effect of DYRK1A overexpression on plasma hcy levels in intermediate hhcy, we quantified plasma levels of hcy in  $Cbs^{+/-}$  mice supplemented with methionine with or without *Dyrk1a* gene transfer. As expected, the hcy level in  $Cbs^{+/-}$  mice supplemented with methionine was higher than that of  $Cbs^{+/+}$  mice (Fig. 1b). Interestingly,  $Cbs^{+/-}$  mice supplemented with methionine and injected with AdDYRK1A had a significantly lower plasma hcy level than uninjected  $Cbs^{+/-}$  mice (Fig. 1b).

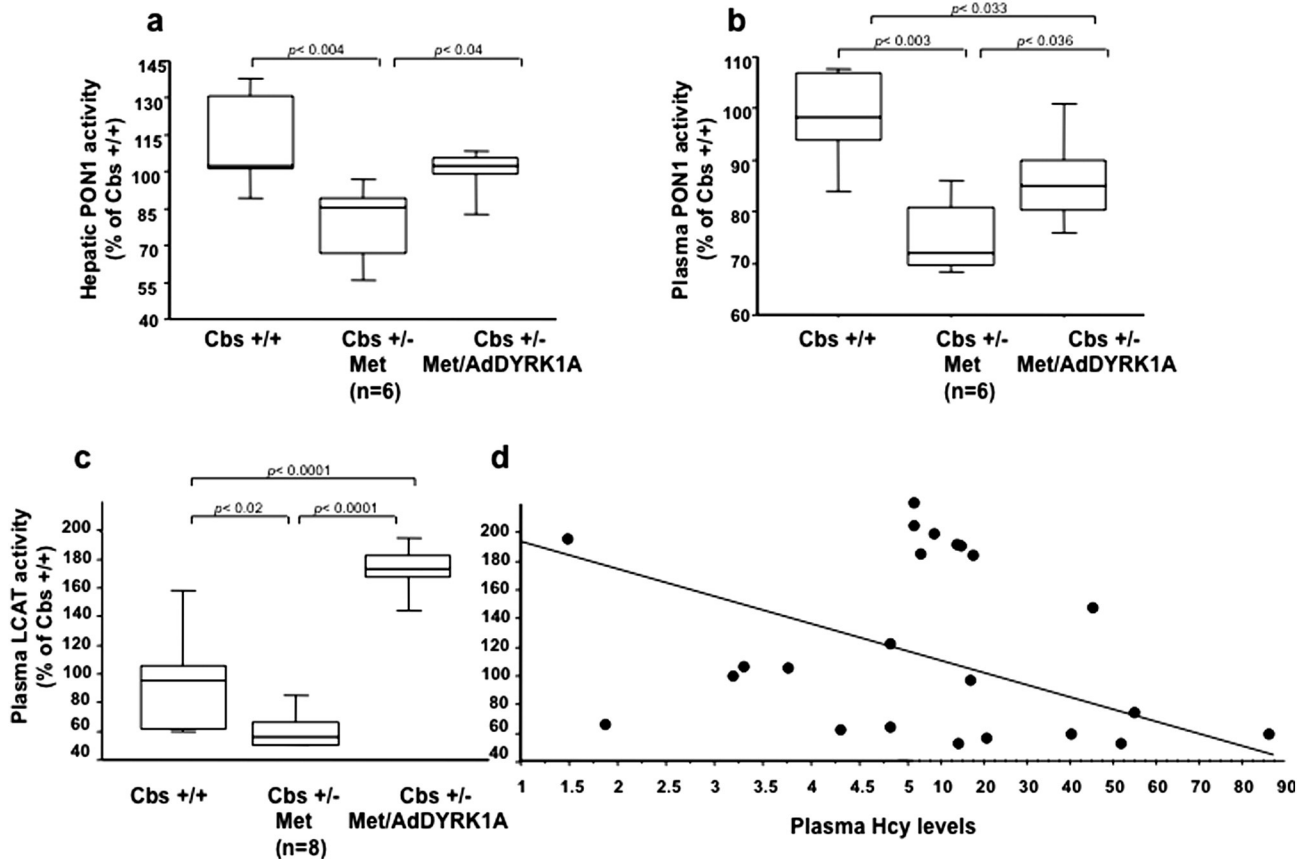
We next studied hepatic activities of the main enzymes implicated in hcy metabolism. Assays of hepatic CBS activity revealed, as expected, significantly lower activity in  $Cbs^{+/-}$  mice supplemented with methionine than in  $Cbs^{+/+}$  mice (Fig. 1c). Injection of AdDYRK1A resulted in a significant increase in CBS activity (Fig. 1c). A Spearman correlation test revealed a significant negative correlation between plasma hcy levels and hepatic CBS activity for hcy levels  $>5 \mu\text{M}$  ( $\rho = -0.861$ ,  $p < 0.0013$ , Fig. 1d), and a significant positive correlation between liver *Dyrk1a* expression and CBS activity ( $\rho = 0.655$ ,  $p < 0.01$ , Fig. 1e). Since hcy is lowered by its conversion into SAH, we also assayed SAHH activity in the liver of mice and found an increase after injection of AdDYRK1A ( $100.2 \pm 9.7$  versus  $146.1 \pm 18.1$ ;  $p < 0.05$ ;  $n = 8$  for

each). We previously reported that increased SAHH activity occurs concomitantly with an increase in NQO1 activity [10,11]. Therefore, we assayed hepatic NQO1 activity to confirm this finding in the intermediate hhcy model. After injection of AdDYRK1A, we observed a significant increase in NQO1 activity ( $100.3 \pm 6.6$  versus  $136.6 \pm 9.7$ ;  $p < 0.01$ ;  $n = 8$  for each).

### 3.2. *Dyrk1a* overexpression reduces plasma ALT and increases kynurenic acid levels in mice with intermediate hhcy

Hepatocellular injury often results in an increase in serum ALT, and serum ALT levels are used as a marker for liver injury. To determine the effect of *Dyrk1a* overexpression on liver damage, we quantified plasma levels of ALT in  $Cbs^{+/-}$  mice supplemented with methionine with or without *Dyrk1a* gene transfer. The mean ALT level in  $Cbs^{+/-}$  mice supplemented with methionine was significantly higher than that of  $Cbs^{+/+}$  mice (Fig. 2a). In contrast, in  $Cbs^{+/-}$  mice supplemented with methionine and injected with AdDYRK1A, ALT level was significantly lower than in uninjected  $Cbs^{+/-}$  mice (Fig. 2a).

Previous results have shown that low concentrations of hcy stimulate production of the tryptophan derivative KYNA, whereas high levels inhibit KYNA formation in vitro and in vivo [21]. Interestingly, KYNA might exert a protective role on multiple organs during heatstroke through anti-inflammatory mechanisms [22]. Here, we found that the plasma levels of KYNA were significantly lower in  $Cbs^{+/-}$  mice supplemented with methionine but trended higher after injection with AdDYRK1A (Fig. 2b). Moreover, a Spearman correlation test revealed a significant positive correlation between plasma ALT and

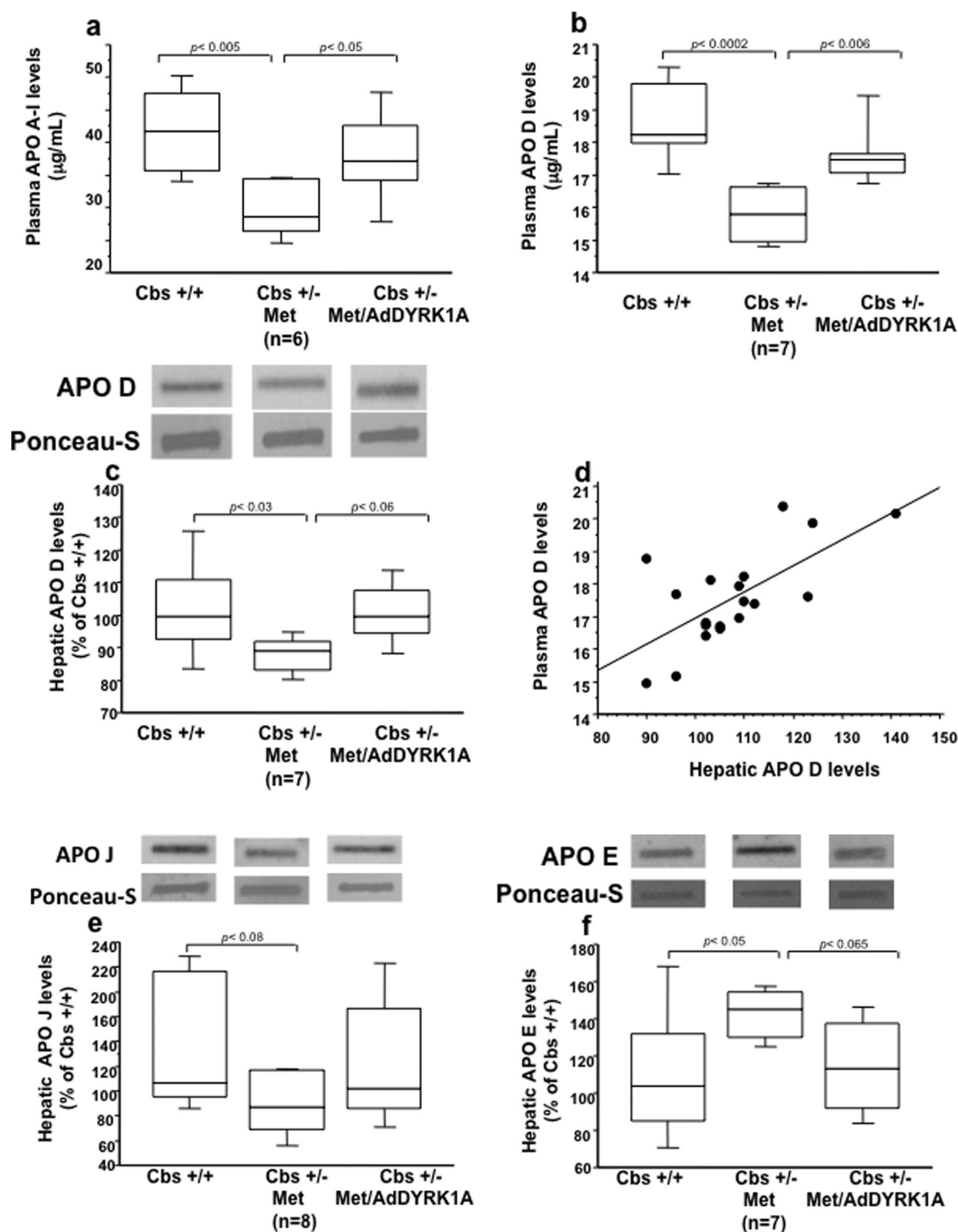


**Fig. 3.** Effect of hepatic overexpression of *Dyrk1a* on PON1 and LCAT activity in mice with intermediate hhcy. Hepatic PON1 activity (a) and plasma PON1 (b) and LCAT (c) activity in wild-type ( $Cbs^{+/+}$ ) mice and  $Cbs^{+/-}$  mice supplemented with methionine and injected ( $Cbs^{+/-}$  Met/AdDYRK1A) or uninjected ( $Cbs^{+/-}$  Met) with AdDYRK1A. Data were normalized to the mean of wild-type mice ( $Cbs^{+/+}$ ). Data correspond to the medians with interquartile ranges.  $n$  = number of mice. Levels of plasma LCAT activity and hcy levels were negatively correlated at  $p < 0.05$  with a  $\rho = -0.423$  (d).

hcy levels (Fig. 2c) and a negative correlation between ALT and KYNA levels (Fig. 2d) ( $\rho = 0.543$ ,  $p < 0.01$ ;  $\rho = -0.563$ ,  $p < 0.05$ , respectively). These results confirm the deleterious effect of hhcy on liver and support a beneficial effect of DYRK1A expression on rescuing the hhcy phenotype.

### 3.3. DYRK1A overexpression rescues enzymes involved in HDL metabolism in mice with intermediate hhcy

PON-1 is an HDL-associated protein that plays a major role in HDL-mediated protection against coronary artery disease [23], and hepatic



**Fig. 4.** Effect of hepatic overexpression of *Dyrk1a* on apolipoprotein levels in mice with intermediate hhcy. Plasma APO A-I (a), APO D (b) levels and hepatic APO D (c), APO J (e), and APO E (f) levels in wild-type (Cbs<sup>+/+</sup>) mice and Cbs<sup>+/-</sup> mice supplemented with methionine and injected (Cbs<sup>+/-</sup> Met/AdDYRK1A) or uninjected (Cbs<sup>+/-</sup> Met) with AdDYRK1A. Hepatic APO D, APO J, and APO E expressions were normalized to the mean of wild-type mice (Cbs<sup>+/+</sup>). Data correspond to the medians with interquartile ranges. n = number of mice. Levels of plasma and hepatic APO D levels were positively correlated at  $p < 0.02$  with a  $\rho = 0.567$  (d).

PON-1 is negatively correlated with plasma hcy levels [24]. PON-1 activity was therefore investigated in the context of hhcy and *Dyrk1a* gene transfer rescue models. As expected, *Cbs*<sup>+/-</sup> mice supplemented with methionine exhibited lower hepatic PON1 activity than *Cbs*<sup>+/+</sup> mice (Fig. 3a). After injection of AdDYRK1A, we observed a significant increase in hepatic PON1 activity (Fig. 3a). Since PON1 is synthesized in the liver and is secreted into the serum, the activity of PON1 was also examined in plasma. *Cbs*<sup>+/-</sup> mice supplemented with methionine had significantly lower plasma PON1 activity than *Cbs*<sup>+/+</sup> mice (Fig. 3b). Injection of AdDYRK1A resulted in significantly higher plasma PON1 activity (Fig. 3b). A Spearman correlation test revealed a significant negative correlation between plasma PON1 activity and plasma hcy as well as ALT levels ( $\rho = -0.631$ ,  $p < 0.01$ ;  $\rho = -0.576$ ,  $p < 0.05$  respectively).

LCAT enzyme converts cholesterol into long-chain cholesteryl esters on HDLs and promotes cholesterol transport from tissues into HDLs. Thus, LCAT is a key enzyme in cholesterol homeostasis and transport regulation [25]. To further investigate the cholesterol-related effects of the *Dyrk1a* gene transfer in hhcy mice, we analyzed the LCAT activity. *Cbs*<sup>+/-</sup> mice supplemented with methionine had significantly lower plasma LCAT activity than *Cbs*<sup>+/+</sup> mice (Fig. 3c). Injection of AdDYRK1A resulted in significantly higher plasma LCAT activity (Fig. 3c). A Spearman correlation test revealed a significant negative correlation between plasma LCAT activity and plasma hcy levels ( $\rho = -0.423$ ,  $p < 0.05$ ) (Fig. 3d).

#### 3.4. *Dyrk1a* overexpression rescues apolipoprotein levels in mice with intermediate hhcy

Because *Cbs*<sup>+/-</sup> mice injected with AdDYRK1A displayed higher plasma LCAT activity, we analyzed plasma APO A-I levels and hepatic expression of apolipoproteins D (APO D) and J (APO J). Each of these apolipoproteins is a component of HDL. APO A-I, the major protein component of HDL, is a co-factor of LCAT. APO D may stabilize LCAT [26]. APO J, or Clusterin, associates with HDL as one of its two main functions [27]. We previously found that plasma APO A-I levels are correlated with plasma hcy level and is increased in moderate hhcy mice injected with AdDYRK1A [10]. As in mice with moderate hhcy, mice with intermediate hhcy exhibited significantly lower circulating levels of APO A-I compared to *Cbs*<sup>+/+</sup> mice (Fig. 4a). Further, AdDYRK1A injection resulted in a significant increase in plasma APO A-I levels (Fig. 4a). A Spearman correlation test revealed a significant negative correlation between plasma APO A-I and hcy levels ( $\rho = -0.569$ ,  $p < 0.02$ ). Consistent with the results for plasma APO A-I, *Cbs*<sup>+/-</sup> mice supplemented with methionine also exhibited significantly lower plasma APO D and hepatic levels of APO D and APO J compared to *Cbs*<sup>+/+</sup> mice (Fig. 4b, c, e), while AdDYRK1A injection resulted in a significant increase (Fig. 4b, c, e). A Spearman correlation test revealed a significant positive correlation between plasma and hepatic levels ( $\rho = 0.567$ ,  $p < 0.02$ ) (Fig. 4d).

APO E, on the other hand, is a polymorphic and pleiotropic apolipoprotein mainly synthesized by the liver and is a component of very low-density lipoprotein (VLDL) and HDL [28]. VLDLs are major contributors to atherosclerosis. *Cbs*<sup>+/-</sup> mice supplemented with methionine exhibited significantly higher hepatic levels of APO E compared to *Cbs*<sup>+/+</sup> mice (Fig. 4f). AdDYRK1A injection resulted in a significant decrease in hepatic APO E levels (Fig. 4f).

#### 3.5. *Dyrk1a* overexpression rescues hepatic I $\kappa$ B levels in mice with intermediate hhcy

The second major function of APO J is to negatively modulate the NF- $\kappa$ B signaling pathway by stabilizing its inhibitor, I $\kappa$ B [27]. We therefore analyzed I $\kappa$ B protein expression in the liver of mice and, commensurate with the decreased levels of APO J, *Cbs*<sup>+/-</sup> mice supplemented with methionine exhibited significantly lower hepatic levels of I $\kappa$ B compared to

*Cbs*<sup>+/+</sup> mice (Fig. 5). Moreover, AdDYRK1A injection resulted in a significant increase in I $\kappa$ B levels (Fig. 5).

#### 3.6. *Dyrk1a* overexpression rescues hepatic Akt/GSK3 signaling pathways in mice with intermediate hhcy

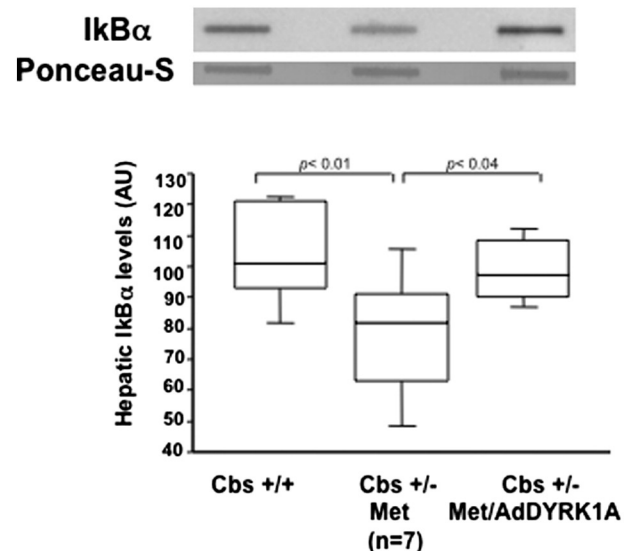
Given the link between DYRK1A and the PI3K/Akt pathway [29,30], we analyzed the activation of Akt in the liver of mice. Commensurate with the decreased DYRK1A protein levels in the liver of *Cbs*<sup>+/-</sup> mice supplemented with methionine, phospho-Akt levels were decreased (Fig. 6a). This decreased activation was rescued following injection of AdDYRK1A to *Cbs*<sup>+/-</sup> mice supplemented with methionine (Fig. 6a).

Akt phosphorylates GSK3 alpha and beta on inhibitory serine residues 21 and 9, respectively. No significant difference was found for GSK3 or phosphorylated GSK3 levels between *Cbs*<sup>+/+</sup> mice and *Cbs*<sup>+/-</sup> mice supplemented with methionine (Fig. 6b, c). However, significantly lower GSK3 (Fig. 6b) and higher phosphorylated GSK3 (Fig. 6c) levels were found in the liver of *Cbs*<sup>+/-</sup> mice supplemented with methionine after injection of AdDYRK1A.

GSK3 phosphorylates cyclin D1 to trigger its degradation. Cyclin D1 levels were similar between *Cbs*<sup>+/+</sup> mice and *Cbs*<sup>+/-</sup> mice supplemented with methionine (Fig. 6d). However, injection of AdDYRK1A induced a significant increase in cyclin D1 level in *Cbs*<sup>+/-</sup> mice supplemented with methionine (Fig. 6d).

#### 3.7. *Dyrk1a* overexpression rescues brain DYRK1A expression in mice with intermediate hhcy

Many studies reported the link between DYRK1A overexpression and cognitive impairment [29]. Moreover, we also found an increase in DYRK1A protein expression in brain of hhcy mice [31,32]. Similar to our previous reports [31,32], mean DYRK1A protein level was increased in brain of *Cbs*<sup>+/-</sup> mice supplemented with methionine when compared with wild-type (*Cbs*<sup>+/+</sup>) mice (Fig. 7). However, after injection of AdDYRK1A to *Cbs*<sup>+/-</sup> mice supplemented with methionine, we observed a decrease in DYRK1A protein levels when compared with *Cbs*<sup>+/-</sup> mice supplemented with methionine but uninjected (Fig. 7).



**Fig. 5.** Effect of hepatic overexpression of *Dyrk1a* on I $\kappa$ B levels in mice with intermediate hhcy. I $\kappa$ B protein expression in the liver of wild-type (*Cbs*<sup>+/+</sup>) mice and *Cbs*<sup>+/-</sup> mice supplemented with methionine and injected (*Cbs*<sup>+/-</sup> Met/AdDYRK1A) or uninjected (*Cbs*<sup>+/-</sup> Met) with AdDYRK1A. I $\kappa$ B expression was determined by slot blotting, and values were obtained by normalization of images from I $\kappa$ B to total proteins colored with Ponceau-S. Data of I $\kappa$ B expression were normalized to the mean of wild-type mice (*Cbs*<sup>+/+</sup>). Data correspond to the medians with interquartile ranges. n = number of mice.

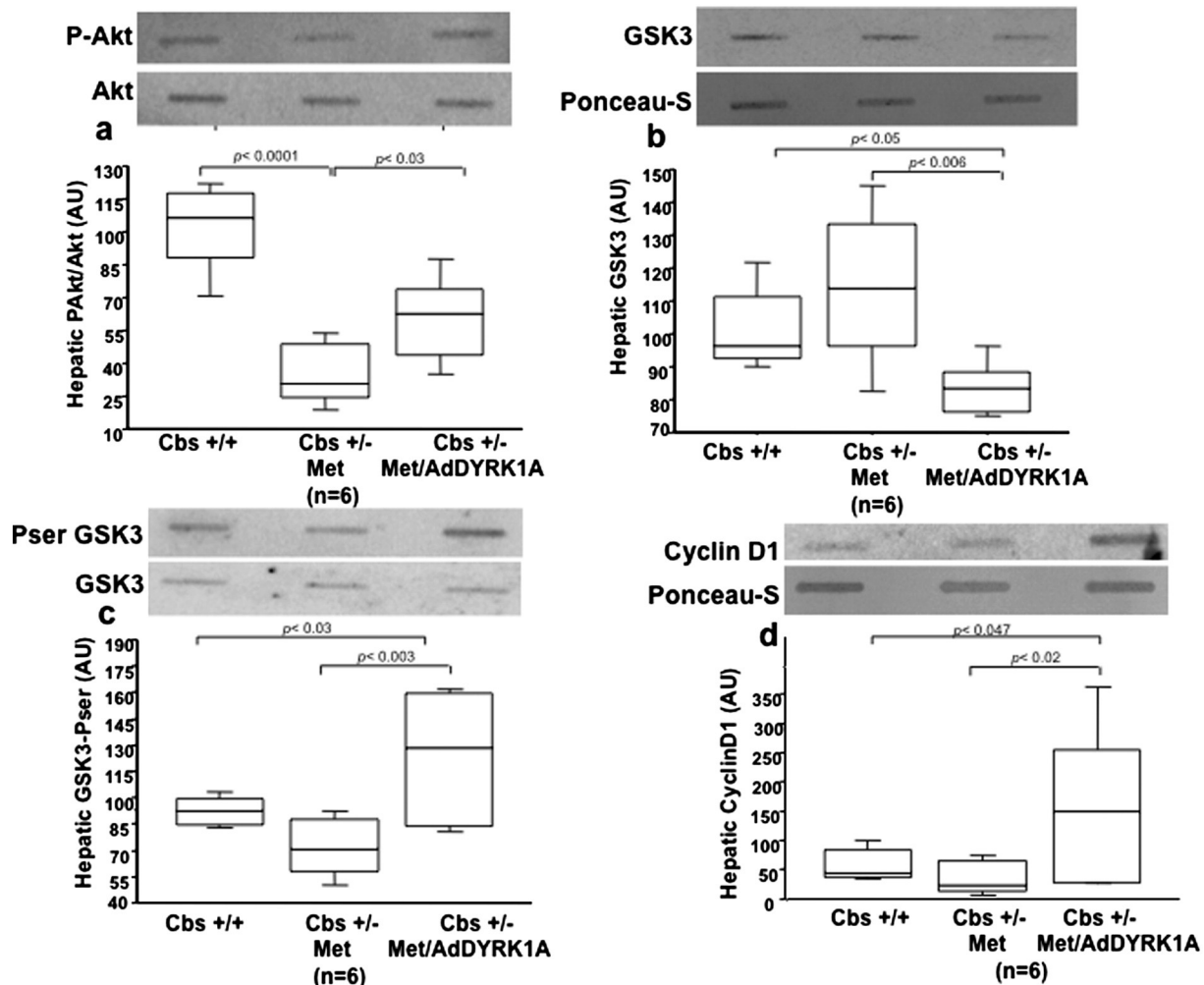
#### 4. Discussion

We recently demonstrated decreased plasma hcy levels in mice with moderate hhcy after adenovirus-mediated *Dyrk1a* gene transfer to hepatocytes [11]. Here, we confirmed this positive effect of DYRK1A expression on plasma hcy levels in mice with intermediate hhcy. Not only the negative correlation between plasma hcy levels and hepatic CBS activity but also the positive correlation between liver *Dyrk1a* expression and CBS activity underline the role of *Dyrk1a* in one carbon metabolism [9,11,12]. Further, the positive correlation identified here between plasma hcy and ALT levels emphasizes the deleterious effect of hhcy on liver function and the beneficial effect of DYRK1A on hepatic hcy metabolism.

Although hcy is produced in the liver, hhcy is a risk factor for cardiovascular diseases [33], and lowering hcy levels could offer a viable approach to preventing cardiovascular diseases. Our findings demonstrate the link between hepatic and vascular functions. We found a negative correlation between ALT and KYNA levels. hcy lowered KYNA production in cultured bovine aortic endothelial cells [34]. Interestingly, KYNA exerts a protective effect against hcy-induced impairment of endothelial cells in vitro [35]. Vascular endothelial cells can also be impaired by lipoproteins, and changes in lipid metabolism and lipoproteins are often observed in cardiovascular diseases. Importantly, a correlation between serum hcy levels and lipids and lipoproteins has been observed [36]. A high concentration of LDL is found in patients with hhcy and primary

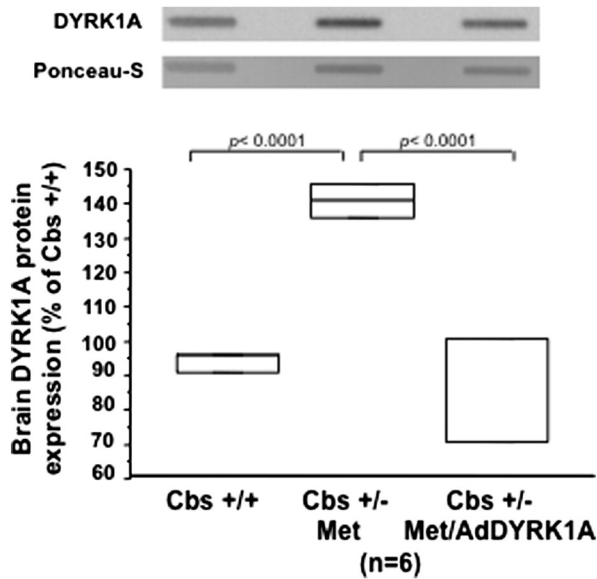
hypertension, coupled to enhanced secretion of VLDL triglycerides [37]. VLDLs and LDLs are considered as one of the most important pro-atherogenic factors, and APO E is a major component of these particles. We found an increased hepatic APO E level in mice with intermediate hhcy, which was abolished after injection of AdDYRK1A. In the vasculature, VLDL and LDL particles are susceptible to oxidative modifications. The COMAC group studies have confirmed the existence of several pro-atherogenic factors, such as the disruption of lipids and lipoprotein balance, for which hhcy intensifies their negative effects on cardiovascular function [38]. Increased hepatic APO E level can also be explained as a compensatory protective effect. Indeed, APO E has a high affinity to LDL receptors on hepatic and extrahepatic cells. In mice, APO E deficiency causes accumulation in plasma of cholesterol-rich VLDL remnants for which prolonged circulation is atherogenic [39]. Conversely, the increased synthesis of hepatic APO E could increase the synthesis of APO E-rich VLDL, and those remnants could be cleared more rapidly due to their increased affinity for LDL receptor.

We found decreased hepatic APO D and plasma APO A-I levels in mice with intermediate hhcy, which was abolished after injection of AdDYRK1A. APO D and APO A-I are components of HDL particles. The observed decreases in APO D and APO A-I may be responsible for the reduced plasma LCAT activity also observed in mice with intermediate hhcy. Moreover, the decreased LCAT activity was abolished after injection of AdDYRK1A. Indeed, APO A-I is a cofactor of LCAT activity, and



**Fig. 6.** Effect of hepatic overexpression of *Dyrk1a* on phospho-Akt, GSK3, phospho-GSK3, and cyclin D1 levels in mice with intermediate hhcy. Phosphorylation of Akt (a), GSK3 alpha and beta expression (b), phosphorylation of GSK3 alpha and beta on ser 21 and 9 (c), and cyclin D1 expression (d) in the liver of wild-type (*Cbs*<sup>+/+</sup>) mice and *Cbs*<sup>+/-</sup> mice supplemented with methionine and injected (*Cbs*<sup>+/-</sup> Met/AdDYRK1A) or uninjected (*Cbs*<sup>+/-</sup> Met) with AdDYRK1A. GSK3 and cyclin D1 expression were determined by slot blotting, and values were obtained by normalization of images from GSK3 and cyclin D1 to total proteins colored with Ponceau-S. Relative protein expression was determined by normalization from p-Akt or p-GSK3 with that of total Akt or GSK3. Data were normalized to the mean of wild-type mice (*Cbs*<sup>+/+</sup>). Data correspond to the medians with interquartile ranges. n = number of mice.





**Fig. 7.** Effect of hepatic overexpression of *Dyrk1a* on brain DYRK1A protein expression in mice with intermediate hhcy. DYRK1A protein expression in brain of wild-type (*Cbs*<sup>+/+</sup>) mice and *Cbs*<sup>+/-</sup> mice supplemented with methionine and injected (*Cbs*<sup>+/-</sup> Met/AdDYRK1A) or uninjected (*Cbs*<sup>+/-</sup> Met) with AdDYRK1A. DYRK1A expression was determined by slot blotting, and values were obtained by normalization of images from DYRK1A to total proteins colored with Ponceau-S. Data of DYRK1A expression were normalized to the mean of wild-type mice (*Cbs*<sup>+/+</sup>). Data correspond to the medians with interquartile ranges. n = number of mice.

APO D can stabilize it [40]. LCAT enzymes convert cholesterol into long-chain cholesteryl esters on HDL and promote cholesterol transport from tissues into HDL [25], and thus are key enzymes in cholesterol homeostasis and transport regulation. We found a negative correlation between plasma hcy levels and plasma LCAT activity, and between plasma hcy and APO A-I levels. Mice with intermediate hhcy also had decreased PON1 activity, which was abolished after injection of AdDYRK1A. PON1 is an HDL-associated enzyme that prevents LDL oxidation [23]. We found a negative correlation between plasma PON1 activity and not only hcy but also ALT levels. Taken together, these results emphasize the deleterious effect of hhcy on the synthesis and the maturation of HDLs, and injection of AdDYRK1A can correct the deleterious effects linked to hhcy.

Similarly, APO J levels were lower in mice with intermediate hhcy, but this change was abolished after injection of AdDYRK1A. APO J has two functions, one in associating with HDL, and the other (Clusterin) in negatively modulating NF- $\kappa$ B signaling by stabilizing its inhibitor, I $\kappa$ B [27]. Commensurate with the decreased levels of APO J, mice with intermediate hhcy exhibited significantly lower hepatic levels of I $\kappa$ B, and injection of AdDYRK1A rescued this effect. Therefore, the decreased APO J levels found in the liver of mice with intermediate hhcy could be responsible for hepatic inflammation [41,42], and AdDYRK1A could play an anti-inflammatory role.

We previously showed aortic PI3K/Akt inhibition and GSK3 activation in mice with moderate hhcy [11]. Targeted hepatic expression of AdDYRK1A resulted in lower plasma hcy, and subsequent PI3K/Akt activation and GSK3 inhibition in the aorta of mice, which can prevent endothelial dysfunction [11]. Another study showed that hhcy inhibits hepatic proliferation during liver regeneration and phosphorylation of Akt [43,44]. Since the PI3K/Akt signaling pathway, which is downstream of growth factor receptors, is important in promoting cell survival and proliferation [45], we analyzed the effect of intermediate hhcy and found a decrease in Akt phosphorylation in the liver of mice. Targeted hepatic expression through injection of AdDYRK1A resulted in PI3K/Akt activation and GSK3 inhibition in the liver of mice, consistent with findings in moderate hhcy mice delivered AdDYRK1A. There are two

main stages during liver regeneration: activation of quiescent hepatocytes, which is controlled by cytokines such as TNF- $\alpha$  and IL-6, and progression in the G1 phase of the cell cycle, which is controlled by growth factors and cyclin D1. Cyclin D1 regulates the cell cycle G1/S transition. We found a non-significant decrease in cyclin D1 in the liver of mice with intermediate hhcy, and injection of AdDYRK1A produced increased cyclin D1. Thus, hepatic dysfunction may be explained by cell cycle arrest and impaired liver regeneration in mice with intermediate hhcy. Akt can enhance expression of cyclin D1 through enhancing its translation and inhibiting protein degradation [46]. Thus, the increase in cyclin D1 in the presence of AdDYRK1A is probably due to the increased phosphorylation of Akt.

We previously showed that targeted hepatic expression of AdDYRK1A abolished the decreased aortic DYRK1A protein level in hhcy mice [11]. Here we found that targeted hepatic expression of AdDYRK1A abolished the increased DYRK1A level in the brain of hhcy mice, which may be the result of hcy lowering. DYRK1A has multiple biological functions, and its overexpression can deregulate many genes in embryonic brains of mice [47]. DYRK1A inhibition is also considered as a target in neurodegeneration [48]. Considering the role of DYRK1A in cognitive functions and hhcy as a risk factor for neurodegenerative diseases [49], our results emphasize the fact that the liver constitutes the best appropriate target for DYRK1A gene transfer in case of hhcy.

## 5. Conclusion

Injection of AdDYRK1A can ameliorate intermediate hhcy and its associated hepatic dysfunction. Thus, DYRK1A gene therapy could be useful in the treatment of hhcy in populations, such as end-stage renal disease patients, which are resistant to hcy lowering by B-complex vitamin therapy. Although we found an increased SAHH activity after AdDYRK1A injection, we do not determine if this increase can modify the methylation status, which needs to be explored.

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